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Biofilms in the oral cavity

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Biofilms are aggregations of microorganisms that grow on surfaces usually found in environments that have a continuous supply of moisture. They can be made from a variety of microorganisms that include bacteria, fungi, yeasts, and algae. The surfaces they grow on are found in a large array of conditions, like in hot springs and the depths of the ocean. As long as the environment contains the appropriate amount of moisture and nutrients, then the surface they choose could be natural or man-made. Bacterial biofilms first form when bacteria in the moist environment comes in close contact with a surface. Their attachment to the surface depends on two main forces, van der Waals and electrostatic. The first force brings the bacteria closer to the surface, and the negative charge of the bacteria and the surface increases the chances that the receptors will adhere. After the bacterial cells adhere, they multiply and eventually form an extracellular polymeric substance matrix. This structure is necessary to protect the bacterial colonies, and helps them grow through forming nutrient channels. Another feature of this matrix is that it facilitates the co-aggregation with other microorganisms. This allows the biofilm to become more diverse, and usually more pathogenic.

Candida albicans is the most commonly found yeast that forms biofilms in the oral cavity. Another yeast that is very similar to it is *Candida dubliniensis*. Because of their related genetic makeup, *C. dubliniensis* is often wrongly identified as *C. albicans*. It is known to play a role in oropharyngeal candidiasis in people who are immunocompromised. This yeast is also known to form biofilms, yet information about the structures it forms have not been the subject of previous research. This study examines *C. dubliniensis* biofilms to find out their structure and how they form in immunocompromised subjects. They used 8 strains of the yeast isolated from subjects who were HIV positive. Biofilms were grown on 96-well microtiter plates. They also collected serum and saliva from subjects who were negative for hepatitis B and HIV. These media were used to precondition wells so that biofilms could grow on environments more similar to the oral cavity. To view biofilms with SEM, they were cultured on

polymethylmethacrylate discs, and coated with gold-palladium. CSLM was also used to look at biofilms that were grown on plastic coverslips. Cells were stained using FUN-1 fluorescent cell stain, which does not exhibit differences in metabolic activity. To test for antifungal susceptibility, fluconazole and amphotericin B were used. The results revealed that biofilms were most metabolically active over the first 8 hours, and reached a plateau 24 to 48 hours later. Various fungal structures were observed, like hyphae and germ tubes, within the first 8 hours. Upon maturation they formed into a multilayered complex. Biofilms grown in serum exhibited a greater adhesion than non-serum biofilms. Those grown in saliva also had strong adhesion, but not to the degree of serum biofilms. Microscopy using SEM revealed that yeast cells adhered first, then germ tubes, and hyphae grew upon them. Antifungal susceptibility showed resistance to both agents. They found that *C. dubliniensis* biofilms shared a similar structure to *C. albicans* in that they both contain a mix of filaments and yeast inside an exopolymer. Through CSLM they were able to view hydrated biofilms, which revealed similar microcolony structure as those found in bacterial biofilms. They believe that this shared arrangement could be the best way for nutrient distribution and waste removal. They explain that antifungal resistance is likely due to the exopolymer layer blocking the agents, multiple genes being expressed simultaneously, and lowered cell growth rates. Biofilm formation of this yeast is an important aspect of how it survives in the oral cavity, and knowing more about its structure will help in its control. [1]

A main cause of periodontal disease is from an excessive amount of plaque formation on the tooth surface. There are hundreds of microorganisms that are thought to come together to form these biofilms. When enough layers of colonization are present, then a bacteria called *Porphyromonas gingivalis* is able to adhere to the gums and exert pathogenic effects. This study explores the biofilm structures of the oral cavity for the presence of quorum sensing signal molecules. These specific molecules indicate that bacteria are able to coordinate through phenotype regulation. They tested for this by first culturing fluids from isolated strains obtained from patients with periodontitis. To test for

the ability to perform extracellular auto inducer activity, bacterial species had to be able to express bioluminescence of the marine bacterium, *Vibrio harveyi*. They found that multiple strains they tested were able to accomplish this, and all of them are associated with periodontal disease. Production of bioluminescence by *V. harveyi* is from the auto inducer, AI-2. Bacteria that were able to make this were found to be *Prevotella intermedia, P. gingivalis,* and *Fusobacterium nucleatum*. When compared to other bacterial strains that produce this same auto inducer, they found the opposite in that they don't depend on glucose concentration or growth phases. This study also found that these strains were unable to produce acyl-homoserine lactones, which is a signaling molecule used in quorum sensing. Although the bacteria found in this study were able to produce bioluminescence which demonstrated some aspect of quorum sensing, the significance of this in relation to plaque formation was not found. [2]

Biofilms of the "red complex" are known to be present in periodontal disease. How these bacteria associate with one another has not been a previous topic of research. This study examines how *P. gingivalis* binds to *Treponema denticola* through surface protein interaction. They first cultured bacterial samples, and obtained the outer membrane of *T. denticola* through extraction. Using a 24-well plate, they added the *T. denticola* membrane and *P. gingivalis* fimbriae, then *P. gingivalis* cells after incubation. To separate proteins they used SDS-PAGE and 2-DE, quantification was done using electrochemiluminescense assays. The results show that *P. gingivalis* fimbriae is necessary to bind to the outer membrane of *T. denticola*. Through Edman degradation, they found that the strongest binding protein between the structures was dentilisin. This protein is found on the surface of *T. denticola*, and it interacts with the fimbriae in a very specific manner. Dentilisin is capable of hydrolyzing many human proteins, but this was not observed in its bacterial interaction. They found that the fimbriae can cause an inhibition of aggregation between *P. gingivalis* and *T. denticola*. They conclude the study by saying that a specific section of the structure is suspected to cause this inhibition. More research into this

interaction must be done, because it seems like an important aspect that could be utilized to inhibit the formation of this pathogenic complex. [3]

Previous biofilm research has examined which microorganisms are most prevalent in dental plaque. An important part of biofilm formation are the microorganisms that adhere to the tooth first. Their attachment allows subsequent attachment of even more, possibly more pathogenic microorganisms. This study examines microbial colonization within the first 6 hours of plaque formation. Biofilm and salivary samples were obtained from 7 male and 8 female subjects who all had good oral health. They collected biofilms at 0, 2, 4, and 6 hour periods after cleaning the tooth surface. By using polyvinylidene fluoride membranes, biofilms were collected. Saliva was collected only once, before biofilm collection, and they were processed the same way. Checkerboard DNA-DNA hybridization with 40 probes was used to identify bacterial strains. The results show that all of the strains tested for were present in saliva or biofilms. There were a large amount of microbes found in saliva that were absent in the biofilms. Compared with other time periods, the strains from hour 0 were significantly lower, and resembled those in saliva. They explain this similarity through exposure to saliva after tooth cleaning, and before biofilm collection. These bacterial species are thought to not adhere very well because they aren't observed very much during the later stages of biofilm formation. This represents the selective competition that microbial colonization can exhibit. Another aspect of microbes in biofilm is their ability to resist the mechanisms that they body uses to eliminate them. They mention that many of the early colonizers in this study produce IgA1. This immunoglobulin neutralizes sIgA found in saliva, which is meant to prevent bacterial adhesion to teeth. Microbial adhesion to teeth is determined by protein interactions, and the presence of diverse adhesins that are capable of binding multiple receptors. An important finding of this study is that *Actinomyces* and *Streptococcus* species were seen within the first couple of hours of biofilm formation. After the first 2 hours the *Actinomyces* species decreased, and *S. mitis* and *Streptococcus oralis* became more abundant. Although an explanation for this behavior was

unavailable, they think that it has something to do with adhesion of *Actinomyces* weakening. Multiple studies examining periodontitis have revealed a specific set of bacteria as a likely cause of the disease, termed the “red complex”. These bacteria were also found in this study, they are *T. forsythia, P. gingivalis,* and *T. denticola*. They mention that bacterial species which provide host protection ere also found in early biofilm formation. Some of these belong to the *Actinomyces* and *Streptococcus* species which neutralize lactic acid and produce hydrogen peroxide that limits the survival of more pathogenic bacteria. They believe that early bacterial colonizers bind to the enamel pellicle with the purpose of protecting the oral cavity from diseases. Future research of early biofilm formation could provide a more direct picture of the adhesion process, and explore the non-bacterial biofilms that also form. [4]

Bacterial co-aggregation is an important aspect of biofilm formation in the oral cavity. Understanding the interactions they make will allow researchers to specify structures that can be targeted to inhibit their formation. Previous studies have revealed that *P. gingivalis* uses its fimbriae to interact with multiple surfaces, cells, and other bacterial species. Although *P. gingivalis* aggregation with *S. oralis* has been shown to be inhibited by human glycoproteins, the structures they use to bind have not yet been revealed. This study examines which structure that *S. oralis* uses to bind to the fimbriae of *P. gingivalis*. First they cultured bacterial samples, and used anti-rFimA antibodies with SDS-PAGE to mark which structures interact between the bacteria. They found *S. oralis* rGAPDH to be very similar to an unidentified protein that was binding with the fimbriae. It was revealed that rGAPDH shared many characteristics with the protein, like molecular mass and inhibitory activity. To view its interaction they used phase-contrast microscopy. Other studies have revealed that GAPDH is involved in the colonization of other *Streptococcus* species, which led them to think it serves the same function in the bacteria they studied. They believe that because *P. gingivalis* and *S. gordonii* use the same binding fimbriae, their stabilization could be mediated using the GAPDH from the *S. oralis* interaction. They also mention that

the GAPDH shared DNA homology with other oral bacteria like *S. gordonii* and *F. nucleatum*. This study's findings could lead future research to see how other bacteria utilize GAPDH in their co-aggregation. [5]

One bacteria that is known to co-aggregate with late stage colonizers in periodontal disease is, *F. nucleatum*. Previous studies have revealed that certain polysaccharides are responsible for bacterial co-aggregation as well as binding to host surfaces. This study examines the roles of *P. gingivalis* lipopolysaccharide (LPS) and capsular polysaccharide (CPS) in co-aggregation with *F. nucleatum*. First they cultured bacterial strains, then extracted the polysaccharides using methods from a different study. They also performed ELISA on the polysaccharides to quantify them. The results revealed that both polysaccharides were able to bind to *F. nucleatum* cells. Through examining inhibitors of the bacterial binding, they found a common structure with free hydroxyl groups at the same positions. Some of these inhibitors were found to be raffinose, lactose, and other sugar derivatives. They also found that possible recognition sites on *P. gingivalis* could be related to Gal or Tal. Other studies on *P. gingivalis* have revealed that glucuronic and galacturonic acids make up part of CPS. They ruled out these two structures because they were not observed as inhibitors. Another finding was that the K1 serotype of *P. gingivalis* was unable to co-aggregate with *F. nucleatum*. They believe this is due to there being no free hydroxyl groups at the 3rd position of D-Gal. With no free hydroxyl groups, it is thought that the lectin was unable to recognize LPS and therefore unable to bind. They also found that CPS binding was greater than LPS binding, and the inverse was true of inhibition. This observation brought them to suggest that these polysaccharides undergo different folding patterns when they are in the environment of the oral cavity. Because they were able to find that CPS and LPS are involved in binding *P. gingivalis* to other species, it will allow future research to utilize these structures as new points of emphasis. [6]

The oral cavity is comprised of a variety of surfaces which are perfect for microbial adhesion and biofilm formation. Because each person is different, they have a unique collection of microbes that are specific to them. This study explores the biofilms that form on the oral tissues of the edentulous or

individuals who lack teeth, and on the surface of their dentures. Before this study was done, there had been very few topics regarding biofilms on these surfaces. Participants of this study included 33 males and 28 females, who were all over 20 years old and had been using complete dentures for at least 1 year. Soft tissue microbes were acquired by swabbing multiple locations with MasterAmp buccal swab brushes. The swabbed areas include the tongue, inner cheeks, gum line, and floor/roof of the mouth. Microbes on dentures were collected using curettes on each tooth, and the same swabbing surfaces. Saliva was collected by spitting into a tube. Analysis of samples was performed using checkerboard DNA-DNA hybridization against 41 test species of bacteria. Results show that *Aggregatibacter actinomycetemcomitans* and *P. gingivalis* were existent on the subjects. This was found to be quite significant, because these two pathogens were previously thought to only form on natural teeth. Examining the microbial profile of the surfaces of the tongue revealed that they were different that the other surfaces of the mouth. This was the same finding in studies done with subjects who had natural teeth. Another finding is that the surface of denture palates did not contain nearly as much bacterial colonization as the tongue did. In other studies there has been a strong link between these two bacteria and systemic diseases like nosocomial bacterial pneumonia and atherosclerosis. Because of the findings of this study, it is possible that those with dentures could suffer from these same disease. Previously it was thought that people who wear dentures could not get these systemic diseases because they did not have natural teeth for certain bacterial species to grow on. This study is believed to be the first one that provides an in-depth view of the bacteria found in the oral cavity of people who wear complete dentures. [7]

Previous research of biofilms associated with periodontitis and gingivitis has only been able to identify the shapes of the bacteria that comprise them. This was due to the limitations of electron microscopy, which only allowed them to view the shapes of biofilms. To identify what the biofilms were made of, researchers used fluorescent in situ hybridization (FISH). This showed them that there is an

adhesion between the *Streptococcus* and *Actinomyces* species. Other research has revealed that there are an estimated 700+ species of bacteria over 9 phylum that are in the oral cavity. This study explores the structure of gingival plaque by using rRNA targeted FISH probes for different groups of oral bacteria. Samples in this study were obtained from 4 patients who had been diagnosed with periodontitis and had a large amount of bone loss. Ten teeth total were extracted from the patients, and the teeth were then fixed and dehydrated. Then, the teeth were bisected, decalcified, and analyzed using FISH. The results revealed new parts of biofilm not previously seen during previous in vivo research. They showed that *C. albicans* and *Streptococcus* form "corncob" structures within the supragingival plaque. Bacteria in the intermediate and 4th layers of plaque were identified as *Tannerella forsythia* and *Spirochaetes* respectively. Also revealed were bacteria like *Actinomyces, Fusobacteria,* and *Tannerella* species being part of subgingival plaque. More species were discovered to colonize on top of the biofilms like, *P. gingivalis, P. intermedia,* and *Parvimonas micra*. The last of the findings for this study was a lining of *Synergistetes* species in close proximity to host defense cells, meaning that they could be have a role in biofilm formation. This study provides a more defined model of oral biofilms by revealing the distribution of bacterial species on multiple gingival surfaces. [8]

Microbial survival in the oral cavity is dependent on their ability to multiply without being disturbed. Some bacterial species are able to achieve this through adhering within spaces between teeth and in periodontal pockets. Following microbe colonization on these surfaces is the formation of plaque. Because plaque is composed of multiple microbes embedded within an intracellular matrix, it is classified as a biofilm. Once they calcify, the treatment of the surfaces they cover, and their removal become much more difficult. They are mainly linked to gum disease and tooth decay. Also, they are thought to be associated with other systemic diseases like ear infections, necrotizing fasciitis, osteomyelitis, prostate infections, endocarditis, and cystic fibrosis. Because of the hundreds of different microbial species that can constitute biofilms, there is not one single method to eliminate their

formation. Treatment can depend on the thickness and the location of the biofilm. There are a variety of approaches to control biofilms, these can range from brushing and flossing to the use of various chemicals that can attack specific aspects of the biofilm. The chemicals can be enzymes, antibiotics, phenols, quaternary ammonium compounds, bisbiguanidines, bispyridines, metallic salts, amino alcohols, and surfactants. Such a broad range of chemicals are used because there are many unidentified microorganisms that comprise biofilms. Treatment of more resistant biofilms must be performed by a dentist and involves the use of tools that clean underneath the gum line. Although it is not possible to completely remove all biofilms that constitute plaque in the mouth, it is easy to mediate their growth so they don't become more resistant and difficult to treat. [9]

In this review, they give an overview of biofilm formation in the mouth, and techniques for their analysis. The oral cavity is an excellent environment for biofilm formation. This is because it is constantly saturated, nutrient rich, warm, and has multiple surfaces for adhesion. The surface of the tooth is generally flat, smooth, and provides protection for bacteria from things that try to eliminate them. After cleaning, teeth are coated in a thin film from saliva called pellicle. This film serves as an excellent attachment point for colonization. Bacteria that tend to adhere first are gram positive, then gram negative species. Afterwards, tertiary colonization occurs through anaerobic bacteria coming in on top of the other biofilm layers. This review found that because less than 1% of microorganisms are reproducible, multiple detection methods must be used to identify the diverse range of species in biofilms. The most common analysis of oral biofilms is through DNA-DNA hybridization. It works by obtaining DNA from the biofilm, and hybridizing it with multiple probes for around 40 different species of microorganism. Another technique is called 16S rRNA Gene Sequencing, which works on the basis that most bacteria contain 16S RNA that can be used for identification. Because it uses a database to compare samples, it significantly broadens the possibilities of a match to a species that is not able to be cultured. A technique called, denaturing gradient gel electrophoresis (DGGE), is performed by first

amplifying marker genes with PCR, then denaturing them in a gel. This allows a more detailed view of the biofilm as it pertains to the amount of species present. Another technique, terminal restriction fragment length polymorphism, also works by amplifying marker genes. The amplified sequences undergo digestion, then separation using restriction endonucleases and capillary electrophoresis. This process allows a more diverse analysis of microbial communities, and has been used to observe bacterial saliva, microbial changes post-treatment, and bacterial formation in root-canals. Although this technique is promising, the review states that it has a high cost and requires very specialized equipment. Some other techniques that have been more recently developed are, denaturing high performance liquid chromatography (DHPLC) and pyrosequencing. DHPLC is similar to DGGE in that it uses PCR to amplify DNA, but it then only partially denatures the amplified sequence. This is useful for finding DNA point mutations in the bacteria. Pyrosequencing is another sequencing technique that can also detect resistance to antibiotics. The review mentions that when saliva and supragingival plaque were analyzed with this technique, it showed that there are approximately 19,000 species. This is significantly more than any other study has been able to find, and shows how extremely diverse biofilms can be. [10]

The *Candida* species frequently colonize and cause infection in people who suffer from diabetes mellitus. When people with diabetes also wear dentures, they are found to be at an even greater risk for *Candida* infections. This study examines the occurrence of the *Candida* species in type 2 diabetics, and nondiabetics who don't have denture stomatitis. They used 90 subjects who were completely healthy, 80 subjects who had denture stomatitis, and 40 subjects who were diabetic and had denture stomatitis. To collect samples they swabbed the surfaces of the upper denture, and colonies were grown on various media. The results showed that *C. albicans* was the most common microorganism, being identified in almost 82% of subjects. The next most common microorganisms were *C. glabrata* and *C. tropicalis*, with the latter being more prevalent in subjects with denture stomatitis. They found that subjects with greater degrees of inflammation on the mucous membrane below their dentures had larger amounts of

C. tropicalis. This observation led them to believe that this fungi could be the main contributor to denture stomatitis. Other studies have revealed that it adheres with greater affinity in people wearing dentures than in healthy individuals. This *Candida* species has also been reported to have the ability to form biofilms on multiple surfaces, and produce degradative enzymes. They concluded that subjects with denture stomatitis had mixed biofilms which contributed to their drug resistance. The findings of this study are important in figuring out which bacteria might cause denture stomatitis, and provides information about candida species other than *C. albicans.* Further research on this subject could explore the composition of biofilms in these kinds of subjects. This would provide a more in-depth look at what can be done to treat and eventually eliminate the oral biofilms that contribute to denture stomatitis. [11]

Being able to identify multiple species of microorganisms in an environment that closely mimics what is found in the oral cavity is necessary to be able to fully understand how they work. Previous research has been done on identifying single and multi-species of biofilms using in situ and in vivo methods. The issue with these studies is that the technology used to identify the microorganisms has been limited to approximately 40 bacterial species. Because there are an estimated 700 species of microorganism in the oral cavity, more advanced techniques are needs to be used to form a more complete picture of the biofilms they form. One recently developed technique at the time of this study that solves this issue is called Human Oral Microbial Identification Microarray (HOMIM). This allows the identification of 272 microbial species through reverse-capture rRNA probes. This study examines if a microcosm model is reproducible using a CDC Biofilm Reactor in which biofilm is grown on composite resins. Research in this study involved many components to answer a variety of questions. Samples of plaque and saliva were obtained from 4 adults, and saliva was also obtained from 10 pediatric subjects who were already in a study using HOMIM. To produce the microcosm model, they used the CDC Biofilm Reactor and incubated it aerobically in basal mucin medium. Saliva and plaque samples were used to

grow biofilms on discs made of hydroxyapatite, silorane composite, and methacrylate composite. Separate experiments using saliva and plaque inoculums were performed, and sucrose pulsing was done with the plaque. SEM was used to observe the biofilms, and revealed multilayered structures of multiple bacterial morphotypes. The results from the microcosm model they created show that they were able to produce stable microbial growth. They found that saliva and plaque produced different biofilms, which was expected when compared to similar studies. Because they wanted to mimic the growth of supragingival plaque, they used aerobic incubation. This caused the biofilms to not contain certain species that were found in the inoculum. They believed that some species were lost due to acidification, and that non-mutans streptococci were selected for as a result of the sucrose pulsing experiment. The surfaces they tested revealed no differences, which they believe was due to the setup they used. It allowed free-colonization between surfaces because of the amount of time the discs were left together. They also found common bacterial species between the subjects of the study, but due to the HOMIM system not being able to discern genetic differences, they were unable to utilize this information. This study provides a reproducible environment that allows the testing of biofilm formation on a variety of surfaces and also mimics the environment of the oral cavity. [12]

Previous studies have suggest a link between periodontal and cardiovascular disease, but there has been no solid evidence that directly connects them. Because there are multiple microorganisms that infect periodontal tissue, studies that only examine a single microbe are not showing a complete picture. This study uses a mouse model to see the effects that a polymicrobial infection has on periodontal disease and other organ systems. They performed this by culturing *P. gingivalis, T. denticola,* and *T. forsythia*. The three strains were mixed and infected into the mouths of ApoE mice. After 16 weeks, the mice were euthanized, and various organs and regions of their bodies were harvested. DNA was then isolated from oral plaque, and analyzed with PCR. The serum was analyzed for antibodies of the three bacterial strains, and alveolar bone loss was measured using histomorphometry. They also

analyzed bacterial genomic DNA from the spleen, abdominal aorta, and the heart using PCR. FISH was used to detect bacterial RNA sequences, and serum was analyzed with HPLC. The results showed them that the mice were infected with all three bacteria in their oral cavities. They also found high levels of alveolar bone resorption, showing the presence of periodontitis. Because this study used a polymicrobial model, and previous ones did not, they were unable to compare their findings. Genomic DNA of the three bacteria were also present in multiple organs. They thought this could mean that the bacteria travelled to these systems through the aorta from the infected gingiva. The possibility of a link between the plaque in the aortic arch, and the polymicrobes was strengthened by a positive correlation. Also found was the presence of high levels of serum amyloid A, which is associated with high density lipoproteins and is known to contribute to cardiovascular disease. Due to this study being the first to include a polymicrobial infection in the oral cavity of mice, it lends a new model for future studies to explore this topic. [13]

Infections with multiple microorganism are difficult to control because they are more resistant to treatments. Two species that are commonly seen together in biofilms are *C. albicans* and *S. aureus. C. albicans* alone is a highly virulent pathogen, and so is Methicillin-resistant/susceptible *S. aureus* (MRSA & MSSA). This study examines the interactions of *C. albicans* with MRSA and MSSA, and also evaluates the amount of phospholipase C and aspartyl proteinase in biofilms. The strains of each microorganism were cultured and standardized. Biofilm formation took place within 96-well microplates for single and multiple species. To find out how many cells formed biofilms they counted CFUs after performing serial decimal dilutions and incubation. To measure metabolic mitochondrial activity they used the 2,3-bis reduction assay, then measured absorbance. Biomass of the biofilms was quantified by fixing the biofilms with methanol, then staining with crystal violet and observing optical density. Aspartyl proteinase was measured after a series of buffers and incubation from the fluorimetric EnzCheck protease Assay kit. To measure phospholipase C, they used the Amplex Red phosphatidylcholine assay

kit. Enzyme activity was observed with fluorescence values. SEM was used to observe biofilm structures and microorganism interactions. The results showed them that all of the strains grew individually and in combination, but MSSA grew more significantly in the presence of *C. albicans*. The biomass results revealed that *C. albicans* biofilms both with and without staphylococcus species were much greater than the staphylococcus species alone. Measurement of enzymatic activity showed them that MRSA doesn't produce a response, and mixed or single MSSA produces a greater response than *C. albicans*. This study revealed that the presence of *C. albicans* has a significant effect on the growth of *S. aureus*. They mention that although there are few studies that examine the enzymatic activity of these specific biofilms, the presence of the enzymes produced in this study are known to increase pathogenic activity. The enzymes do this through increasing adherence to buccal cells and causing resistance to fluconazole, which in turn protects *C. albicans*. Because both of the enzymes were made when the microorganism were cultured together, it showed them that these kinds of biofilms are more pathogenic. Another observation they made was that staphylococcal cells almost entirely covered the candida cells, but only in areas of hyphal growth. From this specific biofilm structure they suggested that S. aureus became more resistant to vancomycin. They conclude the study by saying that the resistances that MRSA has would likely not make a difference when bound to *C. albicans*. Some differences were observed with MRSA and MSSA combinations with *C. albicans*, so further research needs to be done to understand more about these relationships. [14]

The mechanism of biofilm formation in the oral cavity is an important aspect to utilize when developing methods for its elimination. This study examines oral biofilms using an in situ model that analyzes their characteristics over a time period. Previous research using the in situ model have been limited to qualitative analysis, and have not been able to provide a good model of biofilms. To collect oral biofilms while they were growing in the subject's mouth, they used acrylic splints that contained hydroxyapatite discs. The participants were 3 males and 7 females, whom all had no oral diseases.

Biofilms formed on the splints, and were evaluated at various intervals from 1 to 96 hours. The samples were analyzed with a confocal laser scanning microscope, a scanning electron microscope, and a transmission electron microscope. DNA was sequenced with an Ion PGM kit, and PCR was used to quantify the biofilm forming cells. The results show that the amount of bacteria and biofilm thickness both increased over a two-step pattern. During the first 12 hours, the number of bacterial cells increased dramatically. There was a slight increase between 12 to 48 hours, then a big increase until 72 hours. However, after that the cells stopped increasing. Biofilm thickness followed a similar pattern with it maximizing after 48 hours, and decreasing after 72 hours. It was determined that the thickness increases first, then the area, then the thickness increases again with a decrease of both dimensions afterwards. They found that biofilms utilize dead cells as a structure to build upon, as evidenced by the mixture of within multiple layers. When comparing the biofilms grown on acrylic splints against ones that grew on teeth, they found no difference in structure. As opposed to previous studies, bacterial diversity decreases over the first 16 hours, then increases afterwards. This is attributed to a reduction of aerobic bacteria over this time period due to a decrease of partial pressure of oxygen. They attribute the increase in biofilm diversity to when it became anaerobic, and anaerobes were able to survive in such an environment and join the biofilm. Like in previous studies, they found that the *Streptococcus* species was the main one during initial biofilm formation. They also found that this species binds effectively to the pellicle of enamel, and utilizes saliva for nutrients. Because the hydroxyapatite discs are made of enamel, they expected *Streptococcus* formation to occur quickly. Further research they would like to perform is on subjects with oral-diseases to see how biofilms form in the presence of other microorganisms. [15]

Oral fungal infections caused by *C. albicans* are most commonly found in elderly and immunocompromised patients. This fungal pathogen can form biofilms on a variety of surfaces in the mouth. When it interacts with *Streptococcus gordonii* in the oral cavity, it becomes much more difficult

to eliminate because the two species work with each other. This study examines biofilms that contain these two microorganisms, using synthetic saliva and basal medium mucin as nutrient sources. Previous research has examined the interaction of these two species before, but they have not used a medium that mimics what is found in the mouth. Synthetic saliva was produced using a mixture from Wong and Sissons. The microorganisms were cultured and grew on the media in 96-well plates. They were then analyzed using bright-field microscopy and underwent antifungal and antimicrobial susceptibility testing. It was found that the synthetic saliva model allowed *C. albicans* to undergo filamentation, which was not seen in previous studies. They also saw a mutualism between the microorganisms, as was expected from other research. Differences in the biofilm structure were observed when compared to biofilms on other growth media. The biofilms of *S. gordonii* grown on the synthetic saliva were long streptococcal chains instead of dense aggregates. In the mixed biofilms, the media made no difference in terms of interaction, but on the synthetic saliva they had thicker cross sections with larger biomass. To test the antimicrobial and antifungal resistance they used mono/combinational therapy. They targeted biofilms that were forming, and also pre-formed biofilms. The results revealed that single-species biofilms are less susceptible to treatment, and multi-species biofilms are even more resistant. Because of the multiple types of microorganisms that can work synergistically to make biofilms, they are much more difficult to individually target. Treatment for one aspect of the biofilm is basically ineffective, and future research should go into development of treating more than one microorganism at a time. [16]

The enamel pellicle from saliva that forms on cleaned tooth surfaces is responsible for protection from acids and physical damage, but also makes microbial colonization easier. It is thought that a different enamel pellicle can form from gingival fluid that mixes with saliva. This study examines the protein mixtures of saliva and serum using hydroxyapatite adsorption analysis. Also examined are the differences between pellicles formed on various surfaces. Samples were collected from a human donor for the in vitro serum study, and for the in vivo saliva study. Analysis was performed using SDS-

PAGE, liquid chromatography tandem mass spectroscopy, and CyDye imaging. The results show that proteins ae being exchanged at the gingival space. It was determined that salivary proteins in the enamel pellicle are what control its properties. They mention that there are a large amount of phosphorylated proteins in saliva. These proteins are important in their ability to protect enamel. They suggest that proteins that aren't as adsorptive should be replace with ones that are shorter in length. Shorter proteins like casein and statherin would still be able to protect the tooth against decay, and could be applied through direct contact. Another finding is that these shorter proteins could greatly effect biofilm formation, and possibly lead to a decrease in periodontitis incidence. [17]

Biofilm formation on parts of teeth close to the gum line can cause inflammation known as periodontitis. Previous research has not explored if the microbes in these tissues are entirely responsible for other systemic diseases of the heart, endocrine, pulmonary, or joints. This review provides a mechanism of periodontitis, and tries to form a link to rheumatoid arthritis. A large proportion of oral bacteria arise after first tooth formation, mainly due to *Streptococcus salvarius*. Other bacterial species colonize as the individual grows, which depend on what they eat and how they treat their teeth. People who do not suffer from periodontitis have been shown to have a balance between their immune system and oral flora. Biofilm forms after teeth are cleaned and coated in a pellicle. The pellicle allows bacterial adhesion because it changes the charge on the tooth surface which provides recognition sites for bacteria. Species from the *Actinomyces* and *Streptococcus* families are among the first to bind due to their protein recognition capabilities. Other species aggregate with them, and a biofilm is produced. Some bacteria that could not adhere directly to the pellicle can now bind on top of these aggregations, and build up the biofilm. One such bacteria is *Corynebacterium*, a bridge-species, which attracts other bacteria that cause periodontitis. The persistence of dental plaque causes further inflammation due to even more species binding. Some of these are known to be gram-negative anaerobes, which disrupt the balance of normal oral microbes and the immune system. Periodontitis causes a space to form between

the gingiva and tooth, called a pocket. In these pockets, the concentration of oxygen is greatly reduced, and anaerobic microorganisms are able to thrive. Three species found in larger pockets that can also cause bleeding are *P. gingivalis, T. forsythia*, and *T. denticola.* The immune system sends out polymorphonuclear leukocytes to these pockets, which results in further tissue damage. The association of periodontitis with systemic diseases is based on shared lifestyle choices and genetic predisposition. A link they found between the two, was after periodontitis treatment the other diseases improved. One of these diseases that is thought to be more common in periodontitis patients is, rheumatoid arthritis. An explanation they provide is through the pathogenic autoimmunity brought about from oral disease. Before rheumatoid arthritis develops, formations of antibodies against citrullinated peptide antigens (ACPA) are a marker for it. The review states that ACPA binding to osteoclasts can exacerbate pre-existing synovial inflammation, and eventually lead to arthritis development. Although it's inconclusive as to a definite link between oral disease and other systemic diseases, there is enough of a basis for further studies to explore this topic. [18]

Research done on biofilms that form in the oral cavity is relatively new. There are many aspects that have yet to be explored. Because there are so many different biofilms that can form, it will take a great length of time before effective treatments that completely control the adverse characteristics are produced. It seems to be difficult to draw a line between the positive and negative features of biofilms as it relates to oral health. Some bacterial species can protect the surfaces of teeth, but they can also increase pathogenicity through adhering to other microorganisms. A large amount of research has focused on revealing the structures and identification of only a relatively small number of microorganisms that colonize in the oral cavity. Future studies will have numerous resources at their disposal, and the technology for replicating the oral environment will become better. This will allow species that were once unable to be cultured to be able to do so. As a result, the knowledge of oral biofilms will increase significantly.

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